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Gene therapy for murine glycerol kinase deficiency: Importance of murine ortholog

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Abstract

A glycerol kinase (Gyk) knock-out (KO) mouse model permits improved understanding of glycerol kinase (GK) deficiency (GKD) pathogenesis, however, early death of affected mice limits its utility. The purpose of this work was to delay death of affected males to investigate thoroughly their phenotypes. An adenoviral vector carrying the human (Adeno-XGK) or mouse (Adeno-XGyk) GK gene was injected into KO mice within 24 h of birth. Adeno-XGK did not change KO mouse survival time despite liver GK activity greater than 100% of wild type. However, Adeno-XGyk improved KO mouse survival time greater than two-fold. These investigations demonstrate that gene replacement therapy for Gyk KO mice is more efficacious using murine Gyk than human GK. These studies expand our understanding of GKD pathogenesis in the murine model, and show that while murine GKD is more severe than in humans, GKD mice have similar metabolic disturbances to affected humans with hypoglycemia and acidemia.
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Keywords: Glycerol kinase deficiency; Gene therapy; Murine models; Glycerol kinase; Adenovirus; Hypoglycemia; Acidemia; Glycerol metabolism

Glycerol kinase (GK in human, Gyk in mouse) is an X chromosome-encoded enzyme that catalyzes the phosphorylation of glycerol to glycerol-3-phosphate [1]. This reaction has been recognized not only in mammals but also in various bacteria, fungi, and protozoa. Their amino acid sequences are highly conserved throughout evolution with ~50% sequence identity between mammals and bacteria [1–3]. GK in mammals is expressed at highest levels in liver and kidney, but is also expressed at lower levels in brain, intestinal mucosa, adipose tissue, and skeletal and cardiac muscles [1–4].

GK deficiency (GKD) in humans occurs as part of an Xp21 contiguous gene syndrome, complex GKD (cGKD), or as a consequence of mutations within the *GK* gene, isolated GKD (iGKD) [1,5–10]. cGKD has variable association with Duchenne muscular dystrophy and/or adrenal hypoplasia congenita. iGKD includes phenotypes ranging from asymptomatic hyperglycerolemia to symptomatic hyperglycerolemia with vomiting, acidosis, and central nervous system crises [1]. We have shown previously that among GKD patients with point mutations the GK genotype does not predict phenotype [10]. In that study, DNAs from six patients with symptomatic or asymptomatic iGKD were sequenced to identify point mutations, and GK activities were measured in transformed lymphocytes or fibroblasts. The GK activities ranged from a low of 5.53% to a high of

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12.7% of normal, and no correlations were found between levels of GK activity and patients' presence or absence of symptoms.

Gyk knock-out (KO) mice were established by targeted deletion [11]. Liver, kidney, and brown fat GK activities of these KO males were less than 5% of wild type mice. The Gyk KO males had growth retardation by day of life (dol) 2 and died by dol 3 or 4. The biochemical characteristics of mutant males included a >80-fold elevation in plasma glycerol and an approximately 3-fold increase in plasma fatty acid concentrations. The cause of death in the KO mice remained unclear [11], and, therefore, we determined that additional characterization of these Gyk KO males would be helpful to understand better the pathogenesis of the Gyk KO mice and the fidelity of that model with human GKD.

Gene therapy offers the possibility of replacing the deficient gene in the target organ. This approach has been used as therapy for a number of diseases. This therapeutic method may also be helpful in understanding mouse disease models that result in neonatal crisis or neonatal death. Although lipoprotein lipase and argininosuccinate synthetase deficient mice have shorter life spans than Gyk KO mice, gene therapy using recombinant adenovirus was effective in prolonging their survival [12,13].

Our goal was to obtain a better understanding of GK pathogenesis using Gyk KO mice; however, neonatal death limited our investigations. Therefore, the purpose of this study was to provide additional characterization of the Gyk KO mice, and to attempt to moderate their phenotype with gene replacement therapy using adenoviral *GK* and *Gyk* constructs. We observed that the murine Gyk KO model, though more severe than in humans, showed phenotypic fidelity at the metabolic level, with hypoglycemia and acidemia. We also found that the *Gyk* construct was far more efficacious than the *GK* construct, as measured by hepatic GK activities and survival times.

Materials and methods

Experimental animals and genotyping. Gyk KO mice, made using 129/SvJ embryonic stem cells and bred on a C57BL/6J mouse, were obtained from W.J. Craigen at Baylor College of Medicine [11]. They were bred and housed under an Animal Research Committee (ARC) approved protocol at the University of California, Los Angeles (UCLA). All experiments were performed according to a UCLA ARC approved protocol. Newborn mice from carrier females crossed with wild type (WT) males were injected within 24 h of birth with 40 μl of adenovirus via the superficial temporal vein using a 30 gauge needle. At ~45–48 h, or ~118–120 h after injection (dol 3 or 6, respectively), blood was collected and the liver, kidney, brown fat, and heart were harvested.

Identical amounts of Adeno-XGK or Adeno-XGyk virus were injected into KO and WT mice simultaneously. Tail or carcass genomic DNA was collected from mice to determine their genotype. Since in some cases, the carcasses were not recovered post-injection, Tables 2 and 3 show only those mice that had their genotypes confirmed.

Genotypes of Gyk KO mice were determined by PCR. Tail genomic DNA was extracted from injected or sacrificed mice using 2× lysis buffer (Applied Biosystems, Foster City, CA) and proteinase K (Sigma–Aldrich, St. Louis, MO). Primers for genotyping were neo-F2 (5′-gcgcatcgccttctatcgcc-3′) and GykR (5′-gttcaagactccacaccaacc-3′), which amplified the neomycin–Gyk junction fragment and primers for the normal allele, were GykF (5′-gatgccatgaatcgcgactgt-3′) and GykR. The PCR conditions were: 1× KlenTaq buffer, 5μM of neoF and mGykR primers, 2.5 μM of mGykF primer, 0.2 mM of each dNTP, 2.5 U of KlenTaq1 (Ab Peptides, St. Louis, MO), and ~0.1 μg of genomic DNA. The PCR cycling was as follows: 95 °C for 5 min to denature DNA; then 30 cycles of 94 °C for 30 s and 70 °C for 1 min; and a final extension of 72 °C for 5 min.

Assessment of blood gas, blood biochemical data, and urine glycerol level. Blood was collected from the external jugular vein with a pipette tip after the vein was opened with a needle. The collected blood for measuring blood gas and glucose levels was transferred into an Eppendorf tube containing 3 μl of lithium heparin (50 mg/ml) (Sigma–Aldrich). The i-STAT Portable Clinical Analyzer was used with the $^{CG}8^{+}$ cartridge (i-STAT, East Windsor, NJ) to measure pH, HCO_3^-, base excess and glucose from whole blood. The blood for plasma glycerol and free fatty acid analysis was collected and transferred into a 1.5 ml tube containing 3 μl of 0.5 mM EDTA–Na, then centrifuged to separate the plasma. The plasma samples were stored at −80 °C until plasma from 3 to 5 mice could be pooled and assayed at the UCLA Lipid Core. Urine was collected by putting pressure on the lower abdomen of the mice. SIGMA DIAGNOSTIC triglyceride (GPO-Trinder) was used to measure urine glycerol level as per the manufacturer's protocol.

GK assay. Liver, brown fat, heart, and kidney were taken from the injected mice at ~45–48 or ~118–120 h post-injection (dol 3 or 6, respectively). The tissues were transferred into 1.5 ml tubes containing 0.25 M Tris (pH 8.0), homogenized using a pellet pestle (Sigma–Aldrich), and then sonicated for 1 min each, eight times. The whole tissue lysate was centrifuged at 25,000*g* and the supernatant was used to measure GK activity by a radiochemical assay previously described, using 1 μg protein and a 20-min incubation [9]. For each tissue GK activity level, protein from two to three WT mice was extracted and their average activity was used to calculate the relative activity.

Preparation of recombinant adenovirus. The *GK* gene containing the human hepatitis C virus 5′ untranslated region (-cagaccgtgcatc-) [3] was cloned into the pShuttle plasmid (Clontech, Palo Alto, CA) with *Kpn*I and *Not*I sites (pShuttle hGK). The *Gyk* gene containing the 7 base pair sequence upstream from the *Gyk* start site was cloned into the pShuttle plasmid with *Nhe*I and *Xba*I sites (pShuttle Gyk). The fragments between *I-Ceu*I and *PI-Sce*I from pShuttle hGK and pShuttle mGyk were ligated into pAdeno-X adenovirus DNA (Adeno-XGK and Adeno-XGyk) (Clontech), which is a type 5 adenovirus deleted for the E1 and E3 region, according to the manufacturer's protocol. Adenovirus containing the *E. coli* β-galactosidase gene (Adeno-XLacZ) was prepared according to the Adeno-X Expression System (Clontech) manufacturer's protocol. The recombinant adenovirus vectors linearized with *Pac*I were transfected into 293 cells using SuperFect transfection reagent (Qiagen, Valencia, CA). The recombinant viruses were propagated and purified by ultracentrifugation through a $CsCl_2$ gradient then dialyzed with buffer [10 mM Tris (pH 8.0), 2 mM $MgCl_2$, and 5% sucrose (W/V)], and stored at −80 °C until injection. Injected recombinant adenovirus was diluted with phosphate buffered saline (PBS).

Statistical analysis. Data (blood glucose, blood gas analysis, plasma glycerol, and plasma free fatty acid) are shown as means ± standard deviation as determined using the Microsoft Excel Program. The *p* values were determined by *t* test analysis, using the Microsoft Excel Program. For statistical analysis, all samples determined to be unde-

tectable were given the value of 20 mmol/L, representing ⩽20 mmol/L value since iSTAT cannot detect values less than 20.

Results

Blood gas and blood biochemical analyses in untreated mGyk KO mice

To establish baseline values for the Gyk KO mice so that we could evaluate the influence of adenoviral GK/Gyk constructs, we repeated and extended the observations of Huq et al. [11]. Although Gyk KO mice motor activities and behavior were practically identical to the WT and carrier female mice until dol 2, their activity levels were reduced by dol 3. In contrast to the report of Huq et al. [11], we observed that most Gyk KO mice had much less milk in their stomachs compared to WT or carrier female mice. Mouse blood glucose and blood gas levels were measured each day after birth until dol 3 (Fig. 1). On dol 3, compared to WT male and female mice, Gyk KO mice had severe hypoglycemia ($p < 0.01$), metabolic acidemia ($p < 0.05$), and significantly lower HCO_3^- ($p < 0.01$) and base excess (BE) ($p < 0.01$) (Fig. 1). The glucose, HCO_3 and BE in KO mice only differed significantly from WT mice beginning at dol 3 (Figs. 1A, C, and D). KO mouse blood pH levels differed significantly beginning at dol 2 ($p < 0.05$) (Fig. 1B). The WT and carrier female mouse blood glucose levels continually increased from day 1 to day 3. The Gyk KO mice began a trend toward hypoglycemia when they were acidemic on dol 2, and this occurred when they still appeared to have a normal amount of milk in the stomach, suggesting it was due to the metabolic consequences of absent GK activity rather than fasting. There was no significant difference between WT and carrier female mice for blood glucose, pH, HCO_3^- or BE (data not shown).

We also measured plasma glycerol and free fatty acid (FFA) concentrations on dol 3 (Fig. 2). Plasma glycerol

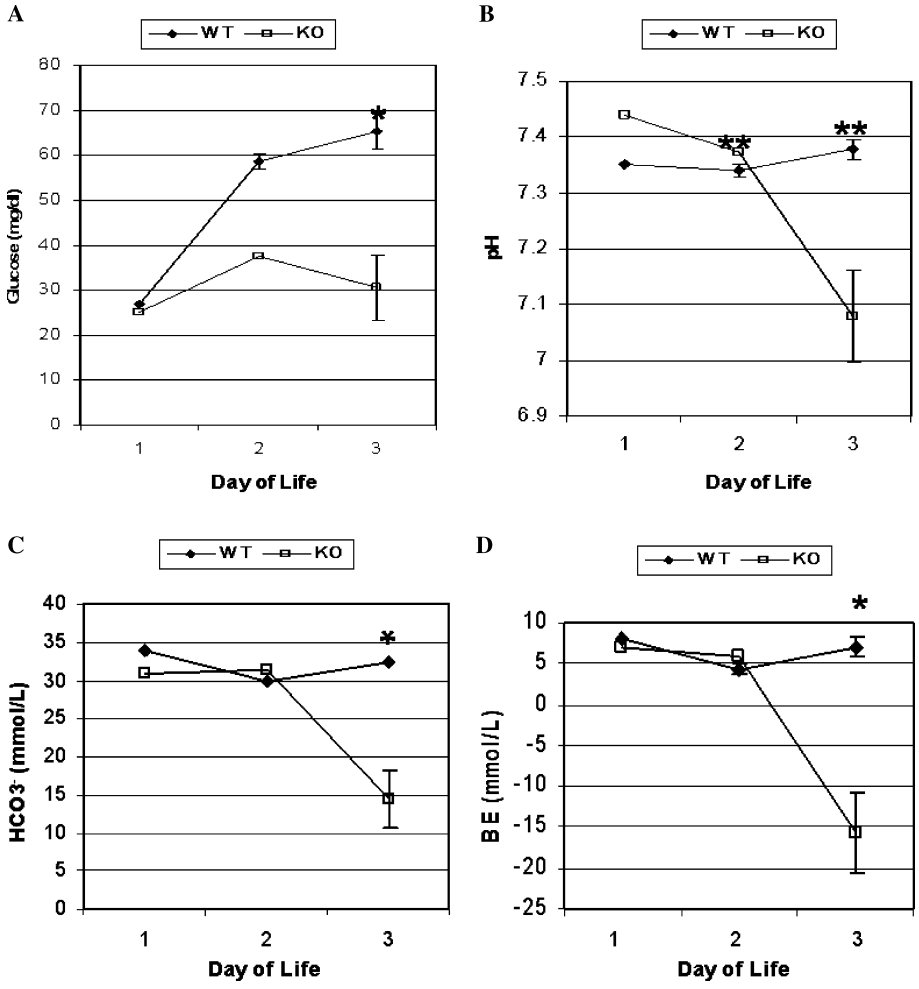

Fig. 1. Blood glucose and blood gas analysis in WT and KO mice. Closed diamonds show WT mice and open squares show KO mice. Animal numbers were as follows: day 1 WT, $n = 1$; KO, $n = 2$; day 2 WT, $n = 7$; KO, $n = 2$; and day 3 WT, $n = 10$; KO, $n = 5$. Data are presented as means ± SD. *Statistically significant ($p < 0.01$) and **statistically significant ($p < 0.05$).

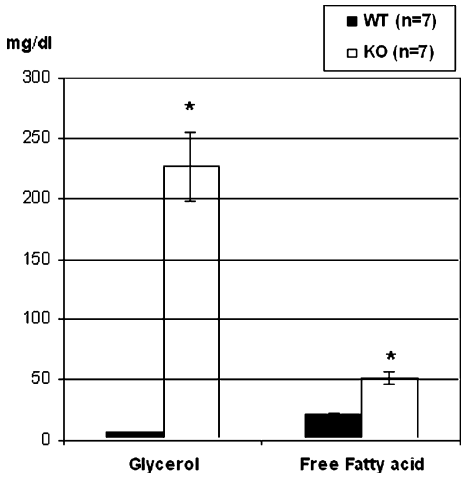

Fig. 2. Plasma glycerol and free fatty acid analysis. Plasma from 4 to 7 mice aged day 2 or 3 (untreated) was pooled for each sample and used for biochemical analysis. Data are presented as means ± SD. The number of pooled samples is shown in parenthesis. *Statistically significant ($p < 0.01$).

levels and plasma FFA from KO mice were significantly higher than WT ($p < 0.01$).

Analysis of the Gyk KO mice treated with Adeno-XGK

In order to investigate GK expression by Adeno-XGK vector in the target organs, dol 1 male mice were injected with 2.1×10^8, 3.0×10^8, 6.0×10^8, and 1.6×10^9 pfu of Adeno-XGK and then were sacrificed at ~45–48 h after injection (dol 3). Liver, kidney, and brown fat were collected and GK activities were measured and expressed as a percentage of GK activity in the corresponding tissue of untreated WT mice. The liver GK activity of Gyk KO mice increased significantly when injected with 3.0×10^8 pfu of Adeno-XGK compared to 2.1×10^8 pfu ($p < 0.01$) and with 6.0×10^8 pfu of Adeno-XGK compared to 3.0×10^8 ($p \leqslant 0.05$) (Fig. 3A). Liver GK activity of KO mice injected with 1.6×10^9 pfu of Adeno-XGK was significantly increased compared to mice injected with either 2.1×10^8 or 3.0×10^8 pfu of Adeno-XGK ($p \leqslant 0.05$). The average liver GK activity of Gyk KO mice was 111 ± 52% of WT, when 1.6×10^9 pfu of Adeno-XGK was injected (Fig. 3B). However, brown fat and kidney GK activities were 0.29 ± 0.05% and 0.27 ± 0.17% of WT brown fat and kidney, respectively, when the mice were injected with 1.6×10^9 pfu Adeno-XGK (Fig. 3B). Of interest, untreated WT mouse kidney and brown fat GK activities were 64.2 ± 9.6% and 37.7 ± 6.8%, respectively, of the untreated WT liver GK activity (data not shown).

In addition, we observed the survival time of mice injected with Adeno-XGK. Untreated KO mice (not injected) did not gain weight and died on or before dol 4 (data not shown), as previously described [11]. The survival time of Gyk KO mice injected with Adeno-XGK was not improved as they all died by dol 4 with a median of dol 3 (Table 1). This is despite a liver GK activity of 111% of WT (Figs. 3A and B).

We measured hepatic GK activity as well as blood HCO_3^-, pH, base excess (BE) and glucose levels with the iSTAT in mice injected with Adeno-XGK ($1.0–1.3 \times 10^9$ pfu) (Table 2). There was no statistically significant improvement in the blood glucose, HCO_3^-, pH, or BE in the Gyk KO mice injected with Adeno-XGK compared to the untreated Gyk KO mice despite an increase in GK activity (Fig. 1 and Table 2). Plasma glycerol concentrations of Gyk KO mice injected with Adeno-XGK remained high and there was no significant difference as compared to untreated Gyk KO mice (data not shown).

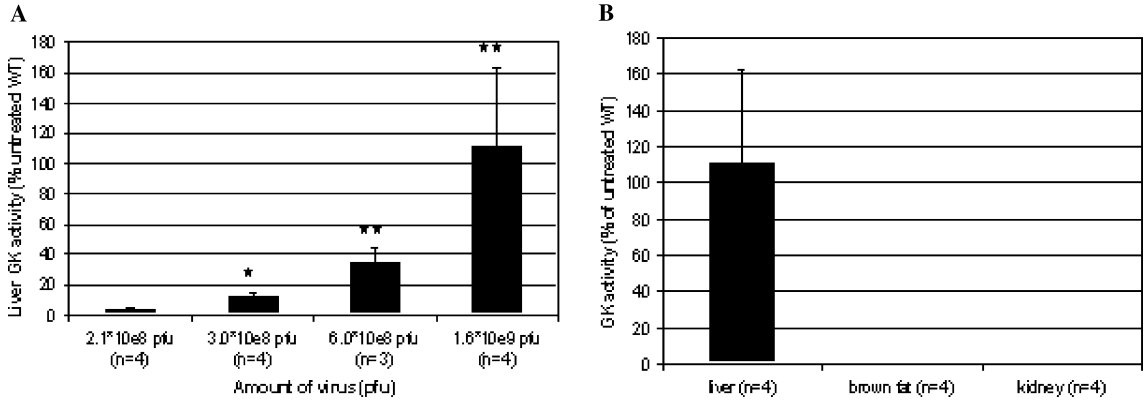

Fig. 3. GK activity in mice treated with Adeno-XGK. (A) Various titers of Adeno-XGK were injected intravenously into KO mice within 24 h of birth and the mice were sacrificed at ~45–48 h after injection. Data are shown as percentage of age matched untreated WT mouse liver GK activity. (B) GK activity in liver, brown fat, and kidney of KO mice injected with 1.6×10^9 pfu of Adeno-XGK. Mice were injected intravenously within 24 h of birth then sacrificed at ~45–48 h after injection. Data are shown as percentage of age matched untreated WT liver, brown fat, and kidney respectively and as means ± SD. Number of mice is shown in parenthesis. *Statistically significant ($p < 0.01$) and **statistically significant ($p < 0.05$).

Table 1
Survival time of mice injected with Adeno-XGK

Injected virus (pfu)	Number of KO mice	Day of death and number of mice				Median age of death
		Day 1	Day 2	Day 3	Day 4	
2.5×10^8	5	2		2	1	3
3.2×10^8	3		1	1	1	3
6.0×10^8	4			4		3
1.3×10^9	3			2	1	3
1.6×10^9	6		2	3	1	3
PBS (control)	7		3	3	1	3

Male mice were injected with the indicated amount of virus within 24 h of birth, and observed every day after injection. If the carcasses were found 3 days after injection, the survival time was determined to be 2 days. *Abbreviations:* pfu, plaque forming unit; PBS, phosphate-buffered saline; KO, knock out.

Table 2
Blood gas and glucose analysis of KO mice treated with Adeno-XGK

Mouse ID #	GK activity in liver (% of WT)	pH	HCO_3 (mmol/L)	BE (mmol/L)	Glucose (mg/dl)
1	5.2	7.202	14	−14	39
2	12.6	7.311	18	−8	<20
3	30.2	7.281	12	−14	<20
4	89.0	7.409	25	1	<20

Each mouse was injected with $1.0–1.3 \times 10^9$ pfu of Adeno-XGK within 24 h of birth and blood was collected at 48 h after injection. Livers were collected from the injected mice to measure GK activity simultaneously. *Abbreviations:* ID, identification; GK, glycerol kinase; WT, wild type; BE, base excess.

Analysis of the Gyk KO mice treated with Adeno-XGyk

Because injection with human GK (AdenoX-GK) did not rescue the Gyk KO mice, we investigated the survival times for mice injected with 3.2×10^8–4.0×10^8 pfu of the mouse Gyk (Adeno-XGyk). Interestingly, Gyk KO mice treated with Gyk survived more than 4 days after birth with a median survival to dol 8.5 (data not shown).

Liver, kidney, and brown fat GK activities in Gyk KO mice injected with Adeno-XGyk were measured. Mice were injected with 3.2×10^8 pfu of Adeno-XGyk within 24 h after birth and sacrificed at ~45–48 or ~118–120 h after injection (dol 3 or 6) to collect tissues and blood. GK activities in liver, kidney, and brown fat of treated mice at dol 3 were $359 \pm 125\%$, $3.92 \pm 1.65\%$, and $2.58 \pm 2.21\%$ of WT, respectively (Figs. 4A and B). Even though only 3.2×10^8 pfu of Adeno-XGyk was injected, liver GK activity in KO mice injected with Adeno-Gyk was statistically higher ($p < 0.05$) than liver of KO mice injected with Adeno-XGK (Figs. 3 and 4), and kidney and brown fat GK activities were also higher in KO mice injected with Adeno-XGyk (kidney, $4.5 \pm 0.3\%$ versus $0.29 \pm 0.14\%$; brown fat, $1.8 \pm 0.5\%$ versus $0.27 \pm 0.09\%$). Although GK activity decreased

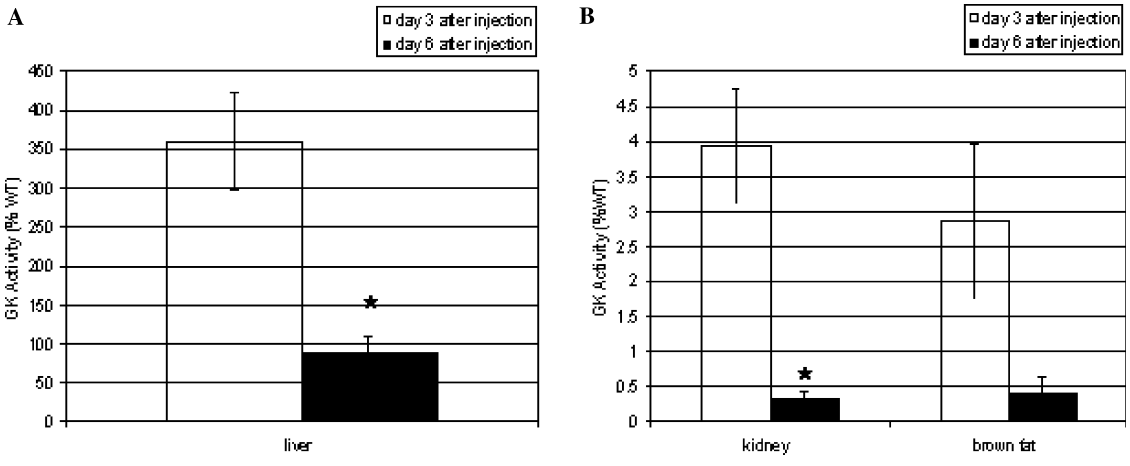

Fig. 4. GK activity from KO mice. 3.2×10^8 pfu of Adeno-XGyk was injected into mice with within 24 h of birth and each tissue was collected at day 3 and day 6 post-injection. Data are shown as percentage of age matched untreated WT mouse GK activity as means ± SEM from (A) liver or (B) kidney and brown fat. ($n = 4$ for day 3 and $n = 3$ for day 6). *Statistically significant ($p < 0.05$) compared to day 3.

Table 3
Blood chemistries of mice injected with Adeno-XGyk

	pH	HCO_3^- (mmol/L)	Base excess (mmol/L)	Glucose (mg/dl)
Dol 3	7.434 ± 0.083 (7.398 ± 0.065)	29.8 ± 1.4 (28.5 ± 2.45)	5.5 ± 2.0 (3.6 ± 3.5)	37 ± 7.9 (37.6 ± 15.5)
Dol 6	7.342 ± 0.023* (7.407 ± 0.018)	26.7 ± 3.1 (29.8 ± 0.84)	0.67 ± 3.7 (5.0 ± 0.3071	42 ± 19.9 (77.2 ± 20.9)

KO and WT mice were injected with 3.2×10^8 pfu of Adeno-XGyk within 24 h of birth and whole blood was collected at day 3 and day 6 after injection. The number in parentheses shows WT. Animal numbers were as follows: day 3, WT $n = 8$, KO $n = 4$; and day 6, WT $n = 5$, KO $n = 3$. Data are presented as means ± SD.
Abbreviation: Dol, day of life.
* Statistically significant ($p < 0.05$).

in KO mice treated with Adeno-XGyk from dol 3 to dol 6, liver GK activity levels remained similar to WT mice, and dol 3 mice injected with Adeno-XGK (Figs. 3B and 4). Kidney and brown fat GK activities were extremely low on dol 6, similar to the mice injected with 1.6×10^9 pfu Adeno-XGK on dol 3 (Figs. 3B and 4).

Blood gas and glucose levels in mice injected with 3.2×10^8 pfu of Adeno-XGyk were measured on dol 3 and dol 6 (Table 3). The HCO_3^-, BE and glucose were not significantly different between WT and treated Gyk KO mice on dol 3 and dol 6. However, the pH on dol 6 was statistically different between treated KO and WT mice ($p < 0.05$) (Table 3). Although mice at day 6 after injection were becoming acidotic, they were better than untreated day 3 KO mice (Fig. 1B). These blood gas and glucose data follow decreasing GK activity in the injected mice between day 3 and day 6 after injection (Fig. 4).

Since Gyk KO mice showed growth retardation [11], we examined body weight with and without Adeno-XGyk treatment (Fig. 5). Adeno-XGK injected GK KO mice that died by dol 4 did not gain body weight (data not shown), and followed a curve similar to untreated KO animals (Fig. 5). However, Gyk KO mice injected with Adeno-XGyk gained body weight, but at a rate consistently less than WT (Fig. 5). The growth retardation of the treated KO mice began on dol 6 and their body weights showed only modest increases from dol 6 to 8 and were significantly different in comparison to WT mice. It is interesting to note that on dol 6 when the Adeno-XGyk treated KO animals ceased to grow, their liver GK activity had fallen to approximately 100% of WT, the activity achieved in the Adeno-XGK animals that also did not grow (Figs. 3 and 4).

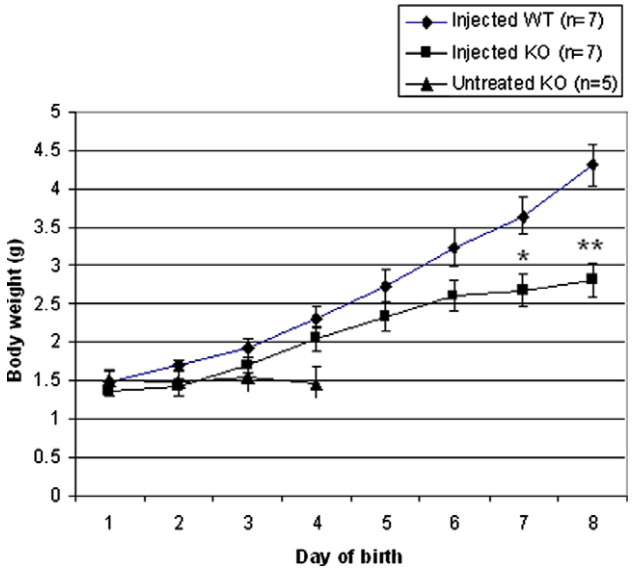

Fig. 5. Body weight of mice over time. 3.2×10^8 pfu of Adeno-XGyk was injected into KO and WT mice within 24 h of birth then their body weight was measured every day. Animal numbers are shown in parentheses. Data are presented as means ± SEM, *statistically significant ($p < 0.05$) and **statistically significant ($p < 0.01$).

Discussion

GK is the first step in the glycerol metabolic pathway and catalyzes the phosphorylation of glycerol to glycerol-3-phosphate [1]. The phenotype of human iGKD includes hyperglycerolemia, hyperglyceroluria, and those with symptomatic iGKD can have vomiting, hypoglycemia, and acidemia [1,5–10,14–16]. Huq et al. [11] showed that Gyk KO mice have hyperglycerolemia, growth retardation, and a uniformly neonatal lethal outcome. We extended the characterization of the phenotypes of Gyk KO mice to provide baseline values to assess efficacy of adenoviral gene therapy. We found that the KO mice had metabolic acidosis, hypoglycemia and hyperglycerolemia, which is similar to patients with the symptomatic form of iGKD. Our data show that Gyk KO mice have lower blood glucose concentrations than WT on day 3 which is in contrast to Huq et al. [11] who showed no statistically significant differences between plasma glucose levels in WT and Gyk KO mice at days 2–3. These differences may be due to the fact that Huq et al. grouped mice of 2–3 days of age, and we separated these two groups; we did not see significant changes on day 2 but did see a statistically significant hypoglycemia on dol 3 (Fig. 1). In addition, our findings are consistent with other mice KO models including the mitochondrial and cytosolic glycerol phosphate dehydrogenase (mGPD and cGPD) KO mice [17]. This is intriguing as these enzymes are immediately down-

stream of GK in glycerol metabolism. mGPD and cGPD KO mice survive longer than Gyk KO mice, but die by days 5–7, and death is preceded by a decreased blood glucose on day 5 [17]. This is strikingly similar to the pattern on our GK mice and shows a common pathway to lethality for contiguous enzymes in this metabolic network.

Gene therapy using viral vectors is one potential treatment for genetic disorders. We attempted to improve the phenotypes of Gyk KO mice using a human *GK* recombinant adenovirus. Liver GK activity of Gyk KO mice injected with Adeno-XGK increased in proportion to the virus titer, and at 1.6×10^9 pfu the Gyk KO liver activity was essentially the same as WT (Fig. 3A). Despite the increase in GK activity, the injected Gyk KO mice had plasma glycerol levels that remained high, and did not live longer compared to their untreated Gyk KO counterparts (Table 2). However, injection with mouse Gyk via Adeno-XGyk briefly rescued the Gyk KO mice as indicated by increased survival time (Table 3). The failure of GK to rescue or modify the phenotype in the Gyk KO mice, despite 111% GK activity in the liver, could be due to the following alternative and not mutually exclusive hypotheses: the loss of GK activity in the liver is not the sole cause of the phenotype; only a fraction of the liver cells are corrected and this is insufficient to ameliorate the phenotype; or human GK is physiologically different from mouse Gyk despite amino acid similarity.

The fact that injection of Adeno-XGK did not rescue the KO mice despite 100% GK activity in the liver strongly suggests that the lethality of this disorder is due, at least in part, to GK deficiency in other tissues. These tissues would be other tissues where GK is expressed such as the kidney, especially given the acidosis and the base deficit in the mice. Liver has the highest Gyk expression; however, in WT mice, the GK activity was significant in the kidney ($64.2\% \pm 9.6\%$ of liver GK activity) and in some individual mice the GK activity was higher in kidney than in liver (data not shown). Kidney GK activity of the mice injected with Adeno-XGK (a titer that gave over 100% liver GK activity) was very low ($0.27\% \pm 0.08\%$ of WT). The low activity in the kidney may be due to difficulties in delivering an exogenous gene into the kidney due to the tissue trophism of the adenoviral vector. This hypothesis would suggest that GK activity may be more important in the kidney than previously thought. In support of this hypothesis, the GK activity was higher in the kidney after injection with Adeno-XGyk ($4.5 \pm 0.33\%$ of WT kidney) and this corresponded with increased blood pH, glucose levels, body weight, and survival time (Fig. 4 and Table 3). Glycerol is a known osmotic diuretic [1] and renal GK is thought to be involved in tubular reuptake of glycerol. Therefore, the Gyk KO mice, which have substantial levels of glyceroluria even after Adeno-XGK (data not shown) are presumably experiencing an osmotic diuresis and possibly other renal effects as a consequence of renal GKD. This speculation would suggest that GK activity in the kidney as well as that in the liver may be crucial for survival.

A second hypothesis to explain why correction to 100% GK activity in the liver did not rescue the KO mice is that only a fraction of the cells in the liver were corrected and that this was insufficient. We would speculate, for example, that gene therapy may have corrected 5% of the cells an average of 20-fold WT liver activity to achieve 100% WT activity. If GKD is a cell autonomous defect, then some threshold cell population would require correction to overcome the hepatic effects of the deficiency, and excess correction of a cell population below that threshold would be inadequate physiologically. Perhaps the improved outcome of Gyk KO mice injected with Adeno-XGyk represents more efficient cellular correction (see below). Other inborn errors of metabolism such as ornithine transcarbamylase deficiency (OTC) have been suggested to be cell autonomous defects [18].

The final and most likely explanation for why Adeno-XGK did not improve the life span of Gyk KO mice is that human GK differs physiologically from mouse Gyk despite variations at only 13 amino acid residues. Therefore, these 13 amino acids may be critical for optimal function in the mouse. To test this hypothesis, Adeno-XGyk was injected into Gyk KO mice. Even with a lower virus titer, the injected mice lived longer than untreated KO mice and Adeno-XGK treated Gyk KO mice (Tables 1 and 3). In addition, the liver GK activity in the mice injected with Adeno-XGyk showed greater than 400% of the WT liver activity suggesting that Adeno-XGK is less efficiently translated or the functional stability of the human GK protein is lower in the mouse. Voltage-dependent anion channels (VDACs), also known as mitochondrial porins, bind several kinases important in metabolism, including hexokinase isoforms and glycerol kinase [1,19,20]. Perhaps this 13 residue difference between GK and Gyk may be important for other functions of Gyk, such as its association with VDACs.

Other differences between the Adeno-XGK and Adeno-XGyk include the sequences upstream of the start sites. Since *GK* does not contain a Kozak consensus sequence, the hepatitis C virus (HCV) 5′ untranslated region (5′UTR) replaced the 5′UTR of hGK [3]. The Adeno-XGyk construct contained the original mouse Gyk 5′UTR. The HCV 5′UTR sequence may have reduced the translation efficiency of GK. This hypothesis could be tested by measuring the amount of protein or RNA in cells infected with Adeno-XGK or Adeno-XGyk. Similar effects were see by Morsy et al. [21] who showed virus containing the human ornithine transcarbamylase (OTC) cDNA failed to express

significantly higher OTC in the recipient animal, and Ye et al. [22] who showed amino-terminal leader peptides of OTC to be important for import of OTC into the mitochondria.

In this study we were able to extend the lives of Gyk KO mice using adenovirus carrying Gyk. We showed that the severe hypoglycemia, metabolic acidemia and growth retardation seen in untreated mice dramatically improved in the Gyk injected mice. However, the maximum survival time was not longer than 9 days after treatment. Some groups tried a second injection after the initial injection for newborn mice and showed that it was effective [13,23]. Walter et al. [23] showed this method did not work in adult mice, but was effective in newborn mice. The effective period of adenoviral (Ad) expression is shorter than adeno-associated virus or lentivirus expression [24,25], but the double injection method will have a longer expression period, which may allow the KO mice to live longer than 9 days. Sun et al. [26] intravenously injected Ad-G6P and adeno-associated virus carrying glucose-6-phosphate (G6P) (AAV-G6P) into neonatal glycogen storage disease type IA (GSD-Ia) mice, then injected AAV-G6P again after 2 weeks. They showed that G6P activity in liver and kidney were 33.1% of WT and 36.2% of WT at 5 months post-injection, respectively, and the Ad and AAV injected mice survival was much longer than the Ad single injected mice. In this method, Ad expressed G6P during the early period after injection, while AAV expressed G6P later and in the kidney. This approach may be useful to improve the survival of the Gyk KO mice. Our goal is to get these mice to weaning so that we can maintain them on a low fat and therefore low glycerol diet with controlled challenges to investigate pathogenesis.

Our data show that the mouse Gyk KO model is similar to the human disease in that the affected males have acidosis, hypoglycemia, and hyperglycerolemia. The mouse model is more severe in that the males die and this may be due to species differences. Our data are consistent with the need to correct GK activity in tissues other than the liver, such as the kidney, and the possibility that GKD is a cell autonomous defect, both of which have implications to gene therapy. Most importantly, our data demonstrate that the design of the adenoviral vector and in particular matching the species from which the construct is derived with the species targeted for therapy will have significant consequences in terms of modulating the phenotype and improving survival. These findings have major implications for the design of human gene therapy vectors for treatment of human genetic diseases.

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